ELSEVIER

Meat Science 57 (2001) 59–64

MEAT SCIENCE

www.elsevier.com/locate/meatsci

A novel polymerase chain reaction (PCR) — denaturing gradient gel electrophoresis (DGGE) for the identification of *Micrococcaceae* strains involved in meat fermentations. Its application to naturally fermented Italian sausages

Luca Cocolin [a,*], Marisa Manzano [a], Daniele Aggio [a], Carlo Cantoni [b], Giuseppe Comi [a]

[a] *Dipartimento Scienze degli Alimenti, Facoltà di Agraria, Università degli studi di Udine, via Marangoni 97, 33100, Udine, Italy*
[b] *Istituto di Ispezione degli Alimenti di Origine Animale, Facoltà di Medicina Veterinaria, Università degli studi di Milano, via Celoria 10, 20121, Milan, Italy*

Received 15 May 2000; received in revised form 1 October 2000; accepted 6 October 2000

Abstract

A new molecular method consisting of polymerase chain reaction (PCR) amplification and denaturing gradient gel electrophoresis (DGGE) of a small fragment from the 16S rRNA gene identified the *Micrococcaceae* strains isolated from natural fermented Italian sausages. Lactic acid bacteria, total aerobic mesophilic flora, *Enterobacteriaceae* and faecal enterococci were also monitored. *Micrococcaceaea* control strains from international collections were used to optimise the method and 90 strains, isolated from fermented sausages, were identified by biochemical tests and PCR-DGGE. No differences were observed between the methods used. The results reported in this paper prove that *Staphylococcus xylosus* is the main bacterium involved in fermented sausage production, representing, from the tenth day of ripening, the only *Micrococcaceaea* species isolated.

Keywords: Lactic Acid; Bacteria; Fermentation; Sausages; Ripening

1. Introduction

The microbiology of dry sausages is varied and complex. The type of microflora that develops during ripening is often closely related to the ripening technique utilized. Sausages with a short ripening time have more lactobacilli, right from the early stages of fermentation and at the end of ripening give an acidic flavour with little aroma. In contrast, sausages with longer ripening times contain higher numbers of *Micrococcaceae* in the early stages of fermentation (Cantoni & Comi, 1986). These have a low rate of acid production and the protease and lipase activity release various aromatic substances and organic acids (Demeyer, Hooze & Mesdom, 1974; Moreno & Kosilkowski, 1973; Virgili, Parolari & Repetti, 1991). The microflora present during the fermentation of raw sausage mixtures consists of *Micrococcaceae* (Bersani, Cantoni & D'Aubert, 1991; del Carmen de la Rosa, Mohino, Mohino & Mosso, 1990; Liepe, 1982; Lucke, 1974; Seager, Banks, Blackburn & Board, 1986; Selgas, Sanz & Ordonez, 1988). Certain *Micrococcaceae* strains have been selected and sold as starter cultures to guarantee reproducible flavour, standardize product properties and shorten ripening times. Studies of the microbial composition of sausages have shown that staphylococci greatly exceed micrococci right from the beginning of fermentation (Bersani et al.; Kloos, Tornabene & Schleifer, 1974; Seager et al.). However, both the microbial genera play a role in the fermentation of sausages as they contribute to colour stability, reduce nitrates to nitrites and break down triglicerides and proteins. The traditional identification methods, which include biochemical tests, are not reliable for separating micrococci from staphylococci (Comi, Citterio, Manzano, Cantoni & de Bertoldi,

* Corresponding author. Tel.: +39-0432-590746; fax: +39-0432-590719.
E-mail address: luca.cocolin@dsa.uniud.it (L. Cocolin).

0309-1740/01/$ - see front matter
PII: S0309-1740(00)00131-5

1992; del Carmen de la Rosa et al.). The variability observed produces a great number of atypical strains. To date these atypical strains have posed a problem as rapid tests have not been available to routinely distinguish staphylococci from micrococci. Tests based on the study of cell wall composition (Schleifer & Kandler, 1992), phylogenetic and chemotaxonomic analysis (Koch & Stackebrandt, 1995; Stackebrandt, Koch, Grozdiak & Schumann, 1995) of the isoprenoid quinones (Collins & Jones, 1981), DNA hybridization (Schleifer, Heise & Meyer, 1979) are too complex for routine use.

In this paper the optimization of a simple and fast molecular method for the identification of micrococci and staphylococci is described. It is based on amplification of the V3 region of the 16S ribosomal DNA (rDNA) and electrophoresis of the polymerase chain reaction (PCR) product in a polyacrylamide gel containing an increasing gradient of denaturants. Due to the differences in the DNA sequence amplified by PCR, it is possible to determine species-specific migration patterns which could be used for specific identification purposes.

2. Materials and methods

2.1. Bacterial control strains

Staphylococcus xylosus DSM 6179, *S. cohnii* susp. *cohnii* DSM 6669, *S. simulans* DSM 20322, *S. intermedius* DSM 20373, *S. carnosus* susp. *carnosus* DSM 20501, *Kocuria kristinae* DSM 20032 and *K. varians* DSM 20033 came from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and were used for the optimisation of the PCR-denaturing gradient gel electrophoresis (DGGE) method.

2.2. Fermented sausage technology and sampling procedures

Fermented sausages were prepared in a local meat factory using traditional techniques. Ingredients (pork meat 66%, lard 33%, NaCl 26 g/Kg, black pepper 1 g/Kg and dextrose, sucrose, ascorbic acid and potassium nitrate mixture 5 g/Kg) were mixed and used to fill natural casings. The first ripening stages involved 6 days drying with the relative humidity increasing from 63 to 75% and a temperature decreasing from 22 to 16°C. The ripening was then carried out for 45 days in store rooms with 75–80% relative humidity and a stable temperature of 13°C. The mix before filling and the fermented sausages at 3, 10, 20, 30 and 45 days were analysed. Three single samples at each step were collected and used for the microbiological analysis.

2.3. pH measurements

Potentiometric measurements of pH were made using a pin electrode of a pH-meter (Radiometer Copenhagen pH M82, Cecchinato, Italy) inserted directly into the sample. Three independent measurements were made on each sample. Means and standard deviations were calculated.

2.4. Microbiological analysis

The samples were subjected to microbiological analysis to monitor the dynamic changes in the population responsible for the ripening of fermented sausages and their hygienic quality. In particular, 25 g of each sample were transferred into a sterile stomacher bag and 225 ml of saline/peptone water (8 g/l NaCl, 1 g/l bacteriological peptone, Oxoid, Italy) were added and mixed for 1 min and 30 s in a Stomacher machine (PBI, Italy). Further decimal dilutions were made and the following analyses were carried out on duplicate agar plates: (1) total aerobic mesophilic flora on Peptone Agar (8 g/l bacteriological peptone, 15 g/l bacteriological agar, Oxoid, Italy) incubated for 48–72 h at 30°C; (2) lactic acid bacteria (LAB) on MRS agar (Oxoid, Italy) incubated with a double layer at 30°C for 48 h; (3) *Micrococcaceae* on Mannitol Salt Agar (Oxoid, Italy) incubated at 30°C for 48 h; (4) *Enterobacteriaceae* on Coli-ID medium (Bio Merieux, France) incubated with a double layer at 37°C for 24–48 h; (5) faecal enterococci on Kanamycin Aesculin agar (Oxoid, Italy) incubated at 42°C for 24 h; and (6) *Staphylococcus aureus* on Baird Parker medium (Oxoid, Italy) with added egg yolk tellurite emulsion (Oxoid, Italy) incubated at 37°C for 24–48 h. After counting, means and standard deviations were calculated. Five *Micrococcaceae* strains from each sample were randomly selected, streaked on brain heart infusion (BHI) agar (Oxoid, Italy) and stored at −20°C in BHI broth containing 30% sterile glycerol, before being subjected to biochemical and molecular identification.

2.5. Biochemical identification

Gram positive and catalase-positive cocci were classified according to Kloos, Schleifer and Gotz (1991) and Kocur, Kloos and Schleifer (1991). Lysostaphin was used to separate staphylococci from micrococci (Schleifer & Kloos 1975). Coagulase activity, urease (Oxoid, Italy), novobiocin resistance (Kloos et al., 1974), detection of DNAse (Tatini, Hoover & Lachica, 1984) and acid production from cellobiose, maltose, mannitol, mannose, raffinose, sucrose and xylose were used to differentiate *Staphylococcus* species, whereas arginine and esculin hydrolysis, acid production from glucose, glycerol and mannose, growth on nutrient agar supplemented with 7.5% of NaCl (w/v) and growth at 37°C were performed to identify *Kocuria* species.

2.6. DNA isolation

A single colony, from a 24 h BHI agar plate incubated at 30°C, was resuspended in 200 μl sterile distilled water and 10 μl proteinase K (25 mg/ml, Sigma, Italy) was added. The DNA was extracted by incubating at 65°C for 1.5 h followed by a step at 100°C for 10 min. Five microlitres were transferred to the PCR reaction mixture after centrifugation at 8000×***g*** for 5 min at 4°C.

2.7. PCR-DGGE protocol

DNA was amplified with primers 338f (5′-ACT CCT ACG GGA GGC AGC AG-3′) and 518r (5′-ATT ACC GCG GCT GCT GG-3′) (Ampe, Ben Omar, Moizan, Wacher & Guyot, 1999) spanning the V3 region of the 16S rDNA. A GC-clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) was attached to the 5′ end of primer 338f. Amplification was performed in a Minicycler (MJ-Genenco, Italy) in a final volume of 50 μl containing 10 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.25 UI *Taq*-polymerase (Roche Diagnostics, Italy), 0.2 μM each primer and the template DNA. In order to increase the specificity of amplification, a touchdown PCR was performed using an annealing temperature of 10°C above the expected annealing temperature of 55°C. The temperature from 65°C was decreased by 1°C each second cycle until the touchdown temperature of 55°C was reached. Ten additional cycles were carried out at 55°C. A denaturation step of 95°C for 1 min was used and extension was performed at 72°C for 3 min with a final extension of 10 min at 72°C which finished the amplification cycle. The PCR product (5 μl) were analysed by electrophoresis in agarose gel.

The Dcode Universal Mutation Detection System (Bio-Rad, CA, USA) was used to analyse by DGGE the PCR products obtained from the strains tested. Electrophoresis was performed in a 0.8 mm polyacrylamide gel (8% [wt/v] acrylamide: bisacrylamide 37.5: 1) containing a 30–60% urea-formamide denaturing gradient (100% corresponded to 7M urea and 40% [wt/v] formamide), increasing in the direction of the electrophoretic run. The gels were subjected to a constant voltage of 130 V for 4 h and 30 min at 60°C and after the electrophoresis, they were stained for 10 min with ethidium bromide, rinsed and photographed using a DS34 Camera system (Polaroid, MO, USA).

3. Results and discussion

The pH evolution and dynamic changes of the monitored populations are shown in Fig. 1. In the first 20 days, the pH of the fermented sausages decreased from 5.73 to 5.18, due to production of acid by LAB which increased from 4.92 to 8.99 $\log_{10}$ CFU/g. *Micrococcaceae* showed a constant increase in cell numbers during the maturation, representing the main population present (8.77 $\log_{10}$ CFU/g) at 45 days. Total aerobic mesophilic flora displayed the highest number at 3 days, then decreased during the next few days of ripening, to reach 5.34 $\log_{10}$ CFU/g at the end of seasoning. After a marked increase in the first 3 days, *Enterobacteriaceae* and faecal enterococci showed a constant reduction due to the decrease of pH which made Gram-negative bacterial growth difficult (Hernandez-Jover, Izquierdo-Pulido, Veciana-Nogues, Marine-Font & Vidal-Carou, 1997). No presumptive *S. aureus* colonies were observed throughout the ripening period. After 20 days of seasoning, an increase in pH was observed, reaching a value of 5.43, explained by the proteolytic activity of the *Micrococcaceae* strains. These strains produce proteases able to induce meat protein degradation resulting in the release of peptides and aminoacids, which neutralise the organic acids produced by LAB in the first stages of the fermentation.

Ninety strains of Gram-positive, catalase positive cocci belonging to *Micrococcaceae* were identified. As shown in Table 1, after biochemical analysis the large majority were defined as *Staphylococcus* spp. and just one strain was represented by *Kocuria* spp. *Staphylococcus* species were identified as *S. simulans*, *S. carnosus* and *S. xylosus*, whereas *K. varians* was the only *Kocuria* species isolated. The distributions of *Staphylococcus* were rather wide in the first 3 days of ripening, characterized by a clear prevalence of *S. xylosus*, which became the only *Micrococcaceae* present from the 10th day of seasoning.

All the isolates were then subjected to molecular identification by the PCR-DGGE method. This procedure is based on electrophoresis of amplified 16S rDNA fragments in polyacrylamide gels containing a linearly increasing gradient of denaturants. In DGGE, DNA

Table 1
Micrococcaceae counts and biochemical identification results from natural fermented sausages during ripening

Days	Counts (CFU/g)	Species	Number of isolates	%
0	3×10^4	*S. simulans*	2	13.33
		S. carnosus	3	20.00
		S. xylosus	9	60.01
		K. varians	1	6.66
3	3.7×10^5	*S. simulans*	3	20.00
		S. carnosus	3	20.00
		S. xylosus	9	60.00
10	2.7×10^6	*S. xylosus*	15	100.00
20	4.6×10^7	*S. xylosus*	15	100.00
30	3.7×10^8	*S. xylosus*	15	100.00
45	6.6×10^8	*S. xylosus*	15	100.00

fragments of the same length but with different base-pair sequences can be separated. Separation in DGGE is based on the electrophoretic mobility of a partially melted DNA molecule in polyacrylamide gels, which is lower than that of the completely helical form of the molecule. The melting of the fragments proceeds in discrete so-called melting domains: stretches of base pairs with an identical melting temperature. Once the melting domain with the lowest melting temperature reaches its melting temperature at a particular position in the DGGE gel, the transition of helical to partially melted molecules occurs, and migration of the molecule will practically cease. Sequence variation within such domains causes their melting temperatures to differ. Sequence variants of particular fragments will therefore stop migrating at different positions in the denaturing

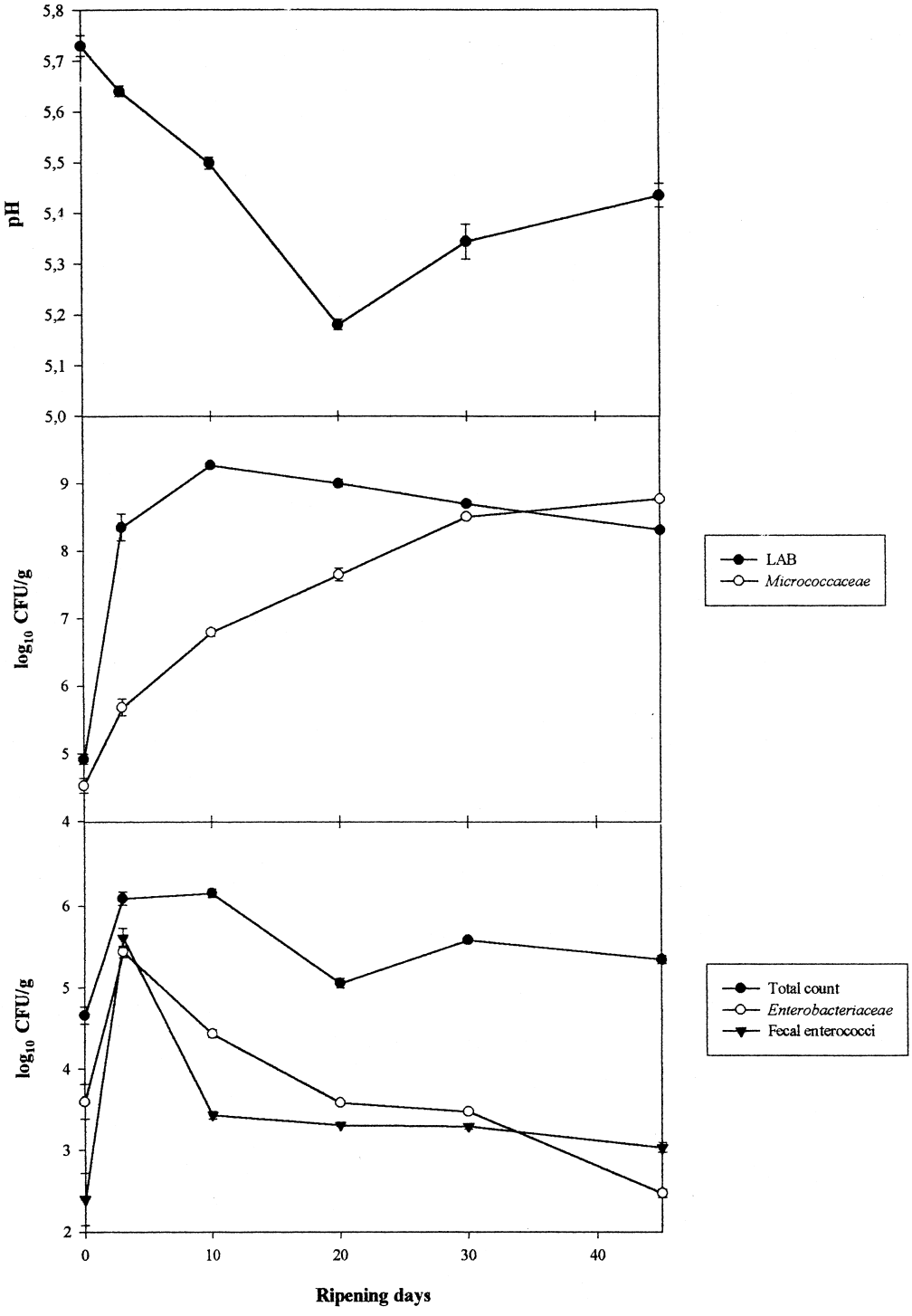

Fig. 1. pH measurements and microbial trends during ripening in the fermented sausages studied.

gradient and hence can be separated effectively by DGGE (Lerman, Fischer, Hurley, Silverstein & Lumelsky, 1984). The bacteria control strains were first used to optimise the protocol which allowed us to differentiate the *Micrococcaceae* population involved in fermented sausage production. As shown in Fig. 2, after DGGE analysis it was possible to define species-specific migrations of the PCR products obtained from the single strain. *Staphylococcus* spp. showed a very different profile in comparison to *Kocuria* spp. which migrated at the bottom of the gel. For all the *Staphylococcus* and *Kocuria* spp. analysed in this study a PCR product of the same molecular weight (about 236 bp) was observed, but it was characterised by differences in the sequence, enabling differentiation. In the DGGE gels, all the species showed a single band, apart from *S. simulans*, which produced four bands probably due to the presence of multicopies of the gene amplified, with sufficient differences to generate different melting domains and in this way different bands in the gel. When the strains isolated from the fermented sausages were analysed by PCR-DGGE, all gave patterns matching the control profiles, making the identification fast and easy. No differences were observed when comparing the results obtained by biochemical identification and the PCR-DGGE method.

The use of the molecular approach proposed in this paper is significant, because of the possibility of avoiding biochemical tests for the identification of *Micrococcaceae* strains isolated from meat. Traditional methods to recognise species are time-consuming and the results obtained, mostly by media colorimetric changes, the evaluation of which is always subjective, are often difficult to interpret. After 8 h the PCR-DGGE is able to give unequivocal results, based on the electrophoretic mobility evaluation of the PCR products obtained from unknown strains, against amplification products generated from the international collection control strains. If new profiles are shown in the DGGE gel, which do not correspond to any control, bands can be cut out of the gel and, after re-amplification, sequenced to determine the closest known relatives of the partial 16S rDNA sequenced obtained, by searching public data libraries (i.e. GenBank) using the BLAST program (Ampe et al., 1999). Moreover the method reported could give a better identification of the strains involved in meat fermentations, avoiding mistakes or difficulties related to the traditional identification methods, especially for the strains selected for their characteristics and used as starters in meat production. However, its future usefulness will depend on accumulating an extensive database to establish that unrelated organisms do not have identical *Micrococcaceae* band patterns.

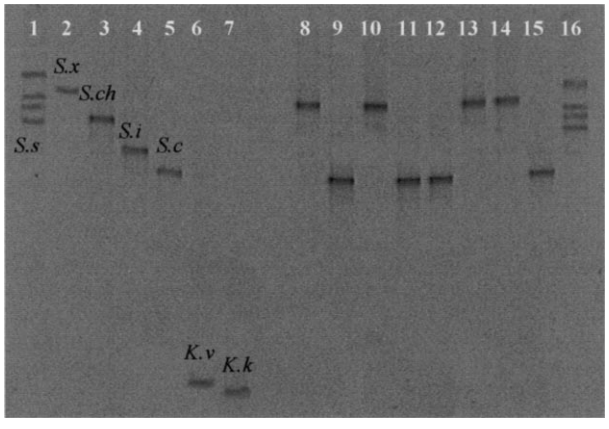

Fig. 2. Denaturing gradient gel electrophoresis profiles of the control *Micrococcaceaea* strains. Lane 1, *Staphylococcus simulans* DSM 20322 (*S.s*); lane 2, *S. xylosus* DSM 6179 (*S. x*); lane 3, *S. cohnii* susp. *cohnii* DSM 6669 (*S.ch*); lane 4, *S. intermedius* DSM 20373 (*S.i*); lane 5, *S. carnosus* susp. *carnosus* DSM 20501 (*S.c*); lane 6, *Kocuria varians* DSM 20033 (*K.v*); lane 7, *K. kristinae* DSM 20032 (*K.k*); lanes 8–10, strains isolated from fermented sausages (lanes 8, 10, 13 and 14 identified as *S. xylosus*; lanes 9, 11, 12 and 15 identified as *S. carnosus*; lane 16 identified as *S. simulans*).

Acknowledgements

This work was supported by the Friuli Venezia Giulia grant L.R. 3/98, art. 16.

References

Ampe, F., Ben Omar, N., Moizan, C., Wacher, C., & Guyot, J. P. (1999). Polyphasic study of the spatial distribution of microorganisms in mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. *Applied Environmental Microbiology, 65*(12), 5464–5473.

Bersani, C., Cantoni, C., & D'Aubert, S. (1991). Osservazioni sugli staphylococci coagulasi-negativi (CNS) presenti negli insaccati crudi stagionati. *Industrie Alimentari, 30*, 12–14.

Cantoni, C., & Comi, G. (1986). I Micrococchi degli insaccati crudi stagionati. *Industrie Alimentari, 25*, 780–782.

Collins, M. D., & Jones, D. (1981). Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. *Microbiological Reviews, 45*, 316–354.

Comi, G., Citterio, B., Manzano, M., Cantoni, C., & de Bertoldi, M. (1992). Evaluation and characterization of *Micrococcaceae* strains in Italian fermented dry sausages. *Fleischwirtshaft, 72*, 1679–1685, 1693-1697.

del Carmen de la Rosa, M., Mohino, M. R., Mohino, M., & Mosso, M. A. (1990). Characteristics of micrococci and staphylococci isolated from semi-preserved meat products. *Food Microbiology, 7*, 207–215.

Demeyer, D., Hooze, J., & Mesdom, H. (1974). Specificity of lipolysis during dry sausage ripening. *Journal of Food Science, 39*, 293–296.

Hernandez-Jover, T., Izquierdo-Pulido, M., Veciana-Nogues, M. T., Marine-Font, A., & Vidal-Carou, M. C. (1997). Effect of starter cultures on biogenic amine formation during fermented sausages production. *Journal of Food Protection, 60*(5), 1–7.

Kloos, W. E., Schleifer, K. H., & Gotz, F (1991) The genus *Staphylococcus*. In A. Balows, H. G. Truper, M. Dworkin, W. Harder, & M.

Schleifer, *The Procaryotes* (2nd ed.; pp. 1369–1420). New York: Springer Verlag.

Kloos, W. E., Tornabene, T. G., & Schleifer, K. H. (1974). Isolation and characterization of micrococci from human skin, including two new species: *Micrococcus lylae* and *Micrococcus kristinae*. *International Journal of Systematic Bacteriology, 24*, 79–101.

Koch, C., & Stackebrandt, E. (1995). Reclassification of *Micrococcus agilis* (Ali-Cohen 1889) to *Arthrobacter* as *Arthrobacter agilis* comb. nov. and emendation of the genus *Arthrobacter*. *International Journal of Systematic Bacteriology 45, 45*, 837–839.

Kocur, M., Kloos, W. E., & Schleifer, K. H. (1991) The genus *Micrococcus*. In A. Balows, H. G. Truper, M. Dworkin, W. Harder, & M. Schleifer, *The Procaryotes* (2nd ed.; pp. 1300–1311). New York: Springer Verlag.

Lerman, L. S., Fischer, S. G., Hurley, I., Silverstein, K., & Lumelsky, N. (1984). Sequence-dermined DNA separation. *Annual Review in Biophysics and Bioengineering, 13*, 399–423.

Liepe, H. U. (1982). Starters cultures in meat production. In H. J. Rehm, & G. Reed, *Biotechnology* (pp. 400–424). Basel: Werlag Chemie.

Lucke, F. K. (1974). Fermented sausages. In B. J. B. Wood, *Microbiology of fermented foods* (pp. 41–49). London: Applied Science Publishers.

Moreno, V., & Kosilkowski, F. V. (1973). Peptides, amino acids and amine liberated from B casein by micrococcal cell-free preparations. *Journal of Dairy Science, 56*, 39–44.

Schleifer, K. H., & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implication. *Bacteriological Reviews, 6*, 407–477.

Schleifer, K. H., & Kloos, W. E. (1975). A simple test for separation of staphylococci from micrococci. *Journal of Clinical Microbiology, 1*, 337–338.

Schleifer, K. H., Heise, W., & Meyer, S. A. (1979). Deoxyribonucleic acid hybridization studies among some micrococci. *FEMS Microbiology Letters, 6*, 33–36.

Seager, M. S., Banks, J. G., Blackburn, C. W., & Board, R. G. (1986). A taxonomic study of *Staphylococcus spp*. isolated from fermented sausages. *Journal of Food Science, 51*, 295–297.

Selgas, M. D., Sanz, B., & Ordonez, J. A. (1988). Selected characteristics of micrococci isolated from Spanish dry fermented sausages. *Food Microbiology, 5*, 185–193.

Stackebrandt, E., Koch, C., Gvozdiak, O., & Schumann, P. (1995). Taxonomic dissection of the genus *Micrococcus: Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend. *International Union of Microbiological Societies, 45*, 682–692.

Tatini, S. R., Hoover, D. G., & Lachica, R. V. F. (1984) Methods for the isolation and enumeration of *S. aureus*. In M. L. Speck, *Compendium of methods for the microbiological examination of food*. Washington, DC:American Public Health Association.

Virgili, R., Parolari, G., & Repetti, L. (1991). Il controllo di qualità nella produzione del salame stagionato. Parte III - Progressi nelle ricerche sulla fermentazione. *Industrie delle Conserve, 66*, 3–8.